AN AUTORADIOGRAPHIC INVESTIGATION OF THE GONADS OF THE PURPLE SEA URCHIN (STRONGYLOCENTROTUS PURPURATUS)[1]

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The duration of the gametogenic events following DNA synthesis by primary spermatocytes and primary oocytes may be determined autoradiographically following the administration of tritiated thymidine. Primary spermatocytes and primary oocytes in premeiotic DNA synthesis are the most advanced germ cells that can incorporate tritiated thymidine. Therefore, by labeling such cells with tritiated thymidine and preparing autoradiograms of gonad samples taken at increasing time intervals thereafter, the labeled cells may be followed as they differentiate into gametes. It is assumed that, before the appearance of labeled gametes, the most advanced labeled cells in any gonad sample have been derived from labeled primary oocytes or primary spermatocytes, and not from labeled gonial cells. By this method, the latter part of oogenesis has been timed in mice (Lima-de-Faria and Borum, 1962; Rudkin and Griech, 1962), while the latter part of spermatogenesis has been timed in rats (Clermont *et al.*, 1959), fruit flies (Chandley and Bateman, 1962), and man (Heller and Clermont, 1963). In the present investigation, the method has been used to study oogenesis and spermatogenesis in the purple sea urchin, *Strongylocentrotus purpuratus*, a marine invertebrate having separate sexes and an annual reproductive cycle. Gonads from urchins collected at monthly intervals throughout one annual reproductive cycle were treated with tritiated thymidine to determine which gonadal cells (both germinal and non-germinal) were synthesizing DNA at each monthly sample. At five different times during the annual cycle, long-term experiments were initiated to time the latter part of gametogenesis.

Materials and Methods

All specimens of *Strongylocentrotus purpuratus* used were collected at low tide from tide pools near Yankee Point, California. Most of the urchins collected weighed between 20 g. and 40 g. and had apparently gone through at least one previous annual reproductive cycle. Samples of 12 urchins were collected at approximately monthly intervals from March, 1963 to February, 1964. On the day of collection, the gonads of the sampled urchins were exposed to tritiated

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thymidine for one hour *in vivo* or *in vitro*. To label gonads *in vivo*, each urchin was injected intracoelomically *via* the peristomial membrane with 0.5 μc. of tritiated thymidine per gram of fresh weight of urchin. Before injection, the aqueous solution of tritiated thymidine obtained from New England Nuclear Corp., Boston (with a specific activity of 2000 mc. per m*M*) was diluted with four parts of sea water. The injected urchin was returned to an aquarium for one hour. Then, one of the five gonads was dissected from the urchin and fixed in sea water-Bouin's fluid. It did not matter which of the five gonads was chosen, since preliminary experiments showed that all acini of all gonads in any individual urchin were

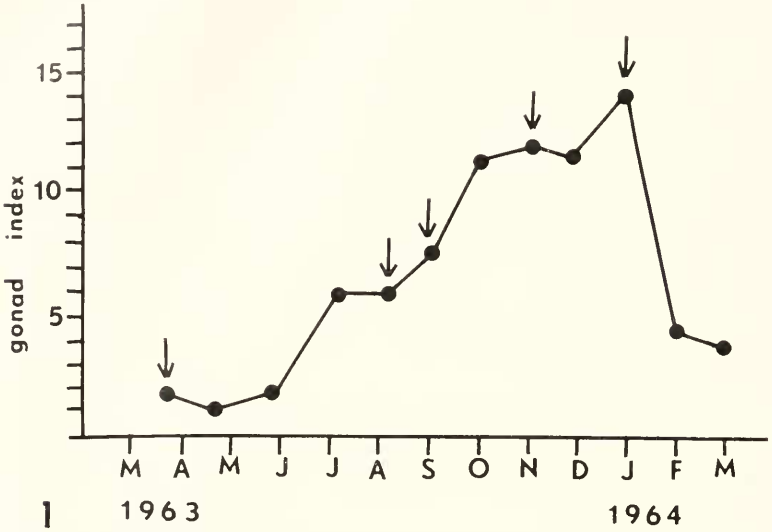

FIGURE 1. The average gonad indices of urchins sampled at approximately monthly intervals during the 1963–1964 annual reproductive cycle. Gonad indices of male and female urchins were considered together for calculation of each point. Long-term experiments were initiated at each of the five points indicated with arrows.

at the same stage of development. From the wet weight of the other four gonads, the gonad index was calculated by the following formula: gonad index $= 1.25 \times \frac{\text{weight of four gonads} \times 100}{\text{weight of urchin}}$. The average gonad index for each monthly urchin sample for March, 1963 to February, 1964 is given in Figure 1.

To label gonads *in vitro*, one gonad was removed from each urchin and placed at once in 5 ml. of 14° C. sea water containing 5 μc. of tritiated thymidine. One hour later, the gonad was fixed in sea water-Bouin's fluid. There was no detectable difference in the number or distribution of radioactive nuclei between the *in vivo*- and the *in vitro*-labeled gonads. *In vitro*-labeling used less tritiated thymidine and resulted in more uniform distribution of tritiated thymidine to the gonadal cells than did *in vivo*-labeling, but at the height of the reproductive season, *in vitro*-labeling resulted in considerable loss of mature gametes from the dissected gonads.

After fixation, the labeled gonads were embedded in paraffin and sectioned at 7 μ and 2 μ. The sections were covered with Kodak AR-10 stripping film; exposure

was for 4, 11 or 25 weeks. The autoradiograms were stained through the emulsion with nuclear fast red (Montruil-Langlois, 1962) or with hematoxylin and eosin. Gonad sections adjacent to those prepared as autoradiograms were always stained with hematoxylin and eosin. In each monthly sample, the number of male and female urchins was approximately equal. The gonads from each month's sample usually showed a fairly wide range of developmental stages. Therefore, after the histology for the whole annual cycle had been studied, each month's gonads were rated as typical, retarded, or advanced. Only typical gonads were used in the description of each monthly sample.

At five different times during the 1963–1964 annual reproductive cycle (indicated by arrows in Figure 1), urchins were collected, injected with 0.5 μc. of tritiated thymidine per gram, and kept in 150-gallon running sea water tanks in the laboratory until sacrificed. In the interval between injection and sacrifice, the urchins were fed as much of the brown alga, *Macrocystis*, as they would eat. At sacrifice, gonad indices were calculated and autoradiograms of labeled gonads were prepared. Of 63 urchins collected and injected on March 22, 1963, 13 were killed after 11 days, 10 after 25 days, 10 after 41 days, 10 after 73 days, 10 after 119 days and 10 after 193 days. Of 19 urchins collected and injected on August 8, 1963, four were killed after 3 days, four after 6 days, four after 9 days, four after 12 days and three after 20 days. Of 31 urchins collected and injected on September 3, 1963, five were killed after 3 days, five after 6 days, five after 12 days, five after 20 days, five after 40 days and six after 100 days. Of 17 urchins collected and injected on November 3, 1963, six were killed after 6 days, six after 20 days and five after 125 days. Of 19 urchins collected and injected on January 1, 1964, six were killed after 6 days, six after 20 days and seven after 65 days.

In addition to the 20-g. and 40-g. adult urchins studied, one group of ten very small urchins, ranging from 0.3 g. to 3.2 g., was collected on June 15, 1963. The gonads of these urchins were too small to be used in calculating a gonad index and too small to dissect from the urchin. Therefore, a cut was made around the ambitus of each urchin to obtain the aboral half of the test with the gonads attached. The gonads were labeled *in situ* for one hour in 5 ml. of 14° C. sea water containing 5 μc. of tritiated thymidine. Fixation in sea water-Bouin's fluid decalcified the tests and permitted sectioning for autoradiography of the entire aboral half of the urchin, including the gonads.

Results

A histological section through an acinus of a sea urchin ovary or testis reveals an outer visceral peritoneum, a middle connective tissue-muscle layer and a lining germinal epithelium. The visceral peritoneum and connective tissue-muscle layer of the gonads of *Arbacia punctulata* were described in detail by Wilson in 1940. In *S. purpuratus*, as in *Arbacia*, the connective tissue-muscle layer is easiest to study in the months after spawning since it becomes stretched very thin and is difficult to see during the ripe season. In the connective tissue-muscle layer of spawned-out gonads, occasional 5 $\mu \times 1$ μ nuclei of fibroblasts were labeled by a one-hour exposure to tritiated thymidine. No labeled nuclei of the smooth muscles of this layer were ever detected. Throughout the year, some of the nuclei of the visceral peritoneum of the gonads become labeled by a one-hour exposure to

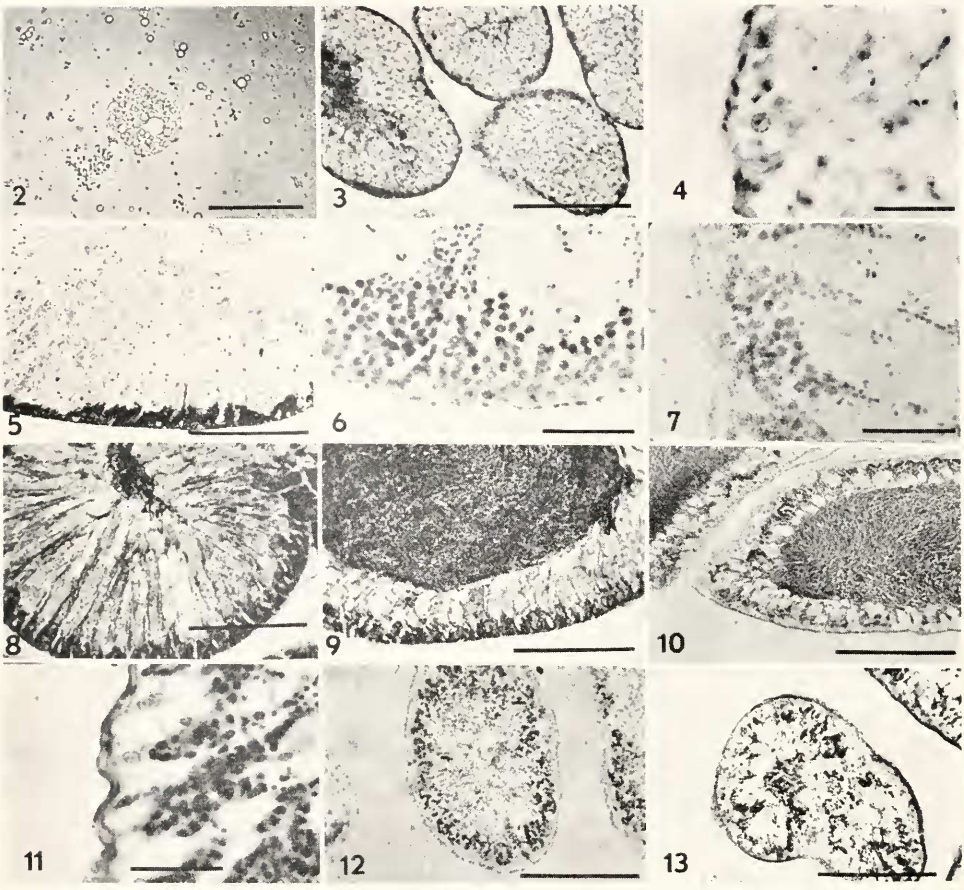

FIGURE 2. A living, globulated nutritive phagocyte in a squash preparation observed by bright-field microscopy. The single vacuole and cytoplasmic globules of reserve are conspicuous. The scale line is 50 μ.

FIGURE 3. A cross-section of acini of a testis from an urchin collected on May 26, 1963. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 4. The periphery of one of the testicular acini of Figure 3. The large nucleus near the base of the germinal epithelium belongs to a primitive spermatogonium, and the smaller nuclei belong to deglobulated nutritive phagocytes. Several relict spermatozoa are also present. The scale line is 20 μ. Hematoxylin and eosin.

FIGURE 5. Part of an acinus of a testis from an urchin collected August 8, 1963. The center of the acinus is at the top of the figure. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 6. The periphery of the testicular acinus of Figure 5. Several trails of spermatids at the top of the figure are proceeding, between the nutritive phagocytes, toward the center of the acinus. Darkly stained spermatocyte nuclei occur along the top border of the basal band of germ cells. At the base of the germinal epithelium are several larger nuclei of primitive spermatogonia. Intermediate in structure and position, between the primitive spermatogonia and spermatocytes, are maturing spermatogonia. The scale line is 25 μ. Hematoxylin and eosin.

FIGURE 7. An autoradiogram of the periphery of the testicular acinus of Figure 5. The base of the germinal layer is toward the left. Some primitive spermatogonia, maturing spermatogonia and spermatocytes are labeled after exposure to tritiated thymidine for one hour. The scale line is 25 μ. Nuclear fast red.

tritiated thymidine. The percentage of labeled peritoneal nuclei is highest in late summer and autumn, when the gonads are increasing in volume. Autoradiograms of gonads from urchins killed several days to several months after injection often show groups of two or more adjacent labeled peritoneal nuclei; each group is probably the progeny of a single initially-labeled cell. These findings support the suggestion of Wilson (1940, p. 472) that "the epithelium accommodates itself to the increasing mass of the gonad by cell division as well as by stretching."

Some workers, such as Caullery (1925), have used the term *germinal epithelium* to refer only to the germinal cells of urchin gonads; there are certain times of year when the germinal cells are widely scattered and not at all organized into an epithelium. This difficulty was avoided in the present investigation by using the term *germinal layer* to include non-germinal as well as germinal cells. At some times of year, the lumen in the center of each acinus may disappear due to encroachment of the non-germinal cells of the germinal layer, but the lumen always reappears as gametes begin to accumulate.

The nutritive phagocytes. The non-germinal cells of the echinoid germinal layer have been described by a number of investigators and have been given several names (see Holland, 1964). The most appropriate name for such cells in *S. purpuratus* is *nutritive phagocyte*. Nutritive phagocytes in the testes and ovaries are similar (aside from differences in the objects they ingest by phagocytosis) and may be discussed together. As Caullery (1925) pointed out, the nutritive phagocytes of echinoid gonads alternate between two morphological phases —globulated and deglobulated. In the globulated phase (July to December of 1963 in the specimens of *S. purpuratus* collected during the present investigation), each nutritive phagocyte is typically a spherical (35 μ in diameter) to oval (45 $\mu \times$ 25 μ) cell containing conspicuous cytoplasmic globules from 1 μ to 12 μ in diameter. A single, large vacuole is often present. Living nutritive phagocytes (Fig. 2) may easily be demonstrated by gently squashing a small piece of gonad in a drop of sea water beneath a coverslip. In gonad sections stained with hematoxylin and eosin, the typical nutritive phagocyte has an oval nucleus 3 $\mu \times 4$ μ which contains a single, poorly-defined nucleolus and little chromatin. The cytoplasmic globules survive fixation and stain strongly with eosin. It is probable that all such globules seen in squash preparations (Fig. 2) come from the cytoplasm of cells disrupted by squashing. Staining with hematoxylin and eosin demon-

FIGURE 8. Part of an acinus of a testis from an urchin collected October 4, 1963. The sperm-filled lumen is at the top. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 9. Part of an acinus of a testis from an urchin collected November 3, 1963. The sperm-filled lumen is at the top. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 10. Parts of two acini of a testis from an urchin collected on January 1, 1964. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 11. The periphery of an acinus of a testis from an urchin collected on January 30, 1964. Several primitive spermatogonia with large nuclei are attaching clumps of maturing spermatogonia and spermatocytes to the base of the germinal epithelium. The scale line is 25 μ. Hematoxylin and eosin.

FIGURE 12. An autoradiogram of a testicular acinus from an urchin collected on January 30, 1964, and exposed to tritiated thymidine for one hour. Many of the maturing spermatogonia and spermatocytes are heavily labeled. The scale line is 150 μ. Nuclear fast red.

FIGURE 13. An acinus of a testis from an urchin collected on February 27, 1964. Clumps of germ cells, detached from the base of the germinal epithelium, are proceeding toward the center of the acinus. The scale line is 150 μ. Hematoxylin and eosin.

strates cells and cellular debris ingested by the nutritive phagocytes; the ingested objects, which cannot be seen in squash preparations, are usually located at the center of an eosinophilic globule. The cell boundaries of the nutritive phagocytes are usually inconspicuous in stained sections of gonad.

In November and December, as the gonad index was approaching its maximum value (1963–1964 annual reproductive cycle), the nutritive phagocytes as seen on the slides begin to change to their alternate morphological state. They shrink to 15 μ in diameter and contain fewer and smaller cytoplasmic globules. Most of the globules shrink to a diameter less than 1 μ or disappear entirely from the testis by December 1, 1963, and from the ovary by January 1, 1964; a month's difference in the time of disappearance of the globules is the only dissimilarity between the nutritive phagocytes of the male and female. No change in nuclear morphology accompanies the loss of globules from the cytoplasm, and the single vacuole is often retained. Deglobulated nutritive phagocytes which retain their vacuoles appear as hollow spheres (usually stuck to one another in clumps) in squash preparations. In stained sections, these vacuolated cells give the germinal epithelium a net-like appearance referred to collectively as the *netzförmigen Syncytium* by Lindhal (1932, p. 382); however, it is likely that they are individual cells and are not organized into a syncytium. In some ripe urchins, especially those that became abnormally ripe due to their failure to spawn in the laboratory, the nutritive phagocytes lose their vacuoles and can only be detected as scattered nuclei in the attenuated germinal layer. Such devacuolated nutritive phagocytes cannot be detected at all in squash preparations. After spawning, the deglobulated nutritive phagocytes remain in their alternate morphological state until the gonad index begins to rise; then cytoplasmic globules reappear.

Autoradiograms of gonads treated for one hour with tritiated thymidine reveal that many of the deglobulated nutritive phagocytes synthesize DNA, presumably in preparation for mitosis. Labeled nuclei of nutritive phagocytes occur at all levels in the germinal layer. Therefore, the production of new nutritive phagocytes is not limited to the base of the germinal layer (as Caullery believed in 1925). Nutritive phagocytes in their deglobulated phase labeled on March 22, 1963 (and their progeny), can still be detected when they have reverted to the globulated state several months after injection. As the gonad index begins to rise and the cytoplasm of each nutritive phagocyte as seen on the slides begins to fill with globules, the number of nutritive phagocytes synthesizing DNA declines. At the height of the reproductive season, no labeled nutritive phagocytes are detected after a one-hour exposure to tritiated thymidine. Thus, the nutritive phagocytes apparently originate from pre-existing nutritive phagocytes dividing by mitosis in the germinal layer of unripe gonads. This conclusion does not agree with earlier schemes deriving the nutritive phagocytes from cells of the visceral peritoneum covering the gonad (Tennent *et al.*, 1931; Miller and Smith, 1931), or from coelomocytes (Liebman, 1950).

Nutritive phagocytes were found in the gonads of all the immature specimens of *S. purpuratus* investigated. In most of these immature gonads, the recognizable nutritive phagocytes observed are vacuolated, but lack eosinophilic globules. Nutritive phagocytes in the gonad of the largest immature urchin (3.2 g) investigated contained a few eosinophilic globules. Exposure of the immature gonads to

tritiated thymidine for one hour labeled many of the nutritive phagocytes. At present, it is not known whether nutritive phagocytes can be found in the gonads of specimens of *S. purpuratus* weighing less than 0.3 g. From the descriptions of Hamann (1888) and MacBride (1903), nutritive phagocytes are apparently not found at the gonadal tube stage and earlier in the life history of the sea urchin. This raises the question of the ultimate source of the nutritive phagocytes. Cuénot in 1892 supplied a possible answer: that germinal cells and nutritive phagocytes both arise from a common primitive cell type very early in the life history of the urchin. These primitive cells may possibly be the *Urkeimzellen* of Hamann (1888) and the "large cells" of MacBride (1903).

The testis. It is most convenient to begin a description of the annual cycle of the urchin testis with the urchins sampled on April 21 and May 26, 1963 (Fig. 3). At this stage of the annual reproductive cycle the germinal layer consists chiefly of vacuolated, deglobulated nutritive phagocytes and the acinar lumens may or may not contain masses of relict spermatozoa, that is, spermatozoa left over from a previous spermatogenesis. Such spermatozoa are usually scattered at all levels in the germinal layer; it is probable that most of them lie within the cytoplasm of the nutritive phagocytes, although this cannot be rigorously demonstrated by light microscopy (Figs. 3 and 4). A few scattered spermatogonia lie at the base of the germinal layer. These spermatogonia, which are usually delimited by a distinct cell membrane, have a small amount of clear or slightly basophilic cytoplasm. Their nuclei are at most about 5 μ in diameter and usually have one or two nucleoli and little chromatin (Fig. 4). In autoradiograms of typical testes from the April 21 and May 26 urchin samples, some of the spermatogonia are labeled by exposure to tritiated thymidine for one hour. DNA synthesis by the spermatogonia is presumably followed by mitotic division. In places at the base of the germinal layer where the spermatogonia have divided several times, they are grouped in cell nests. Such spermatogonia will be referred to hereafter as *primitive spermatogonia.*

The germinal layer of a typical testis from the urchins sampled July 8, 1964, consists mostly of nutritive phagocytes, now containing eosinophilic globules as well as relict spermatozoa; masses of such spermatozoa are still found in a few of the acinar lumens. The germ cell nests at the base of the germinal epithelium contain primitive spermatogonia and also a second type of cell which has a basophilic cytoplasm and a nucleus 3 μ to 4 μ in diameter; the nucleus often lacks a nucleolus and contains more chromatin than the nucleus of a primitive spermatogonium. It is likely that cells of this type are maturing spermatogonia (which may be compared to the intermediate and type B spermatogonia of mammals). A one-hour exposure to tritiated thymidine labels many of the primitive and maturing spermatogonia.

In the typical testes sampled on August 8, 1963, the individual nests of spermatogonia have become confluent, creating a continuous band of germ cells at the base of the germinal layer (Fig. 5). The cells of this band are by now so crowded that individual cell boundaries can rarely be seen even in 2-μ sections. Therefore, it is necessary to describe cell types by nuclear morphology alone. In general, the primitive spermatognia are the most basal cells in the band, and the spermatocytes in the band are closest to the lumen (Fig. 6). The spermatocyte

nuclei are 3 μ to 4 μ in diameter, and some are filled with a thread-like chromatin which stains strongly. Other spermatocyte nuclei contain numerous, stubby chromosomes, which give them a lumpy appearance. There is no reliable criterion for setting apart primary and secondary spermatocytes in *S. purpuratus,* although Nishikawa (1961) reported that secondary spermatocytes contain thinner chromosomes than primary spermatocytes in two Japanese sea urchins. Also in the band of germ cells are numerous cells intermediate in nuclear morphology as well as position between the spermatocytes and basal primitive spermatogonia. Since mitotic figures are often found among these intermediate cells, it is likely that they constitute a population of maturing spermatogonia rather than early primary spermatocytes. As these maturing spermatogonia intergrade structurally with both the primitive spermatogonia and the spermatocytes, it is impossible to establish precise boundaries between these cell types.

In the typical testes sampled in August, a few trails of spermatids arise from the spermatocytes and extend, in the spaces between adjacent nutritive phagocytes, a short distance toward the center of the acinus (Figs. 5 and 6). The more basal, younger spermatids have darkly staining, round nuclei about 2 μ in diameter, while the more mature spermatids have more elongated nuclei, which resemble blunt spermatozoa; spermatid cytoplasm is not demonstrable. A few newly-formed spermatozoa arise from the spermatids in the spaces between adjacent nutritive phagocytes. These spermatozoa have pointed conical heads, 2.5 μ long and 1.2 μ in maximum diameter, and are morphologically indistinguishable from relict spermatozoa in the acinar lumen or in the nutritive phagocytes.

Many primitive spermatogonia, maturing spermatogonia and spermatocytes are labeled by a one-hour exposure to tritiated thymidine (Fig. 7). The labeled primitive and maturing spermatogonia are synthesizing DNA in preparation for mitotic division. It is likely that mitotic divisions of primitive spermatogonia produce both primitive spermatogonia and maturing spermatogonia, while all maturing spermatogonia go through a limited number of mitotic divisions and then differentiate into primary spermatocytes. Thus, the primitive spermatogonia probably constitute the self-sustaining, progenitor compartment and the maturing spermatogonia probably constitute an amplification compartment of germ cell production in the testes. The labeled spermatocytes are presumably pre-leptotene primary spermatocytes (comparable to the resting primary spermatocytes of mammals) synthesizing DNA in preparation for meiotic divisions.

In testes from urchins injected with tritiated thymidine on August 8 and killed three days later, the only labeled germinal cells are primitive spermatogonia, maturing spermatogonia, and spermatocytes. Six days after August 8 injection, the most advanced labeled cells are the younger spermatids. Twelve and 20 days after the August 8 injection, the most advanced labeled cells are spermatozoa. Therefore, the minimum time required in August for a pre-leptotene primary spermatocyte to become a spermatid is between three and six days. This time is probably closer to six days than three days, since only the younger spermatids are labeled at six days. The minimum time required in August for a pre-leptotene primary spermatocyte to become a mature sperm is more than six and less than 12 days. This time is probably somewhat less than 12 days (probably about 10 days), judging from the large number of labeled spermatozoa present 12 days after

injection. In several testes from the August sample, the cytoplasm of the nutritive phagocytes contains many ingested spermatozoa embedded, sometimes singly and sometimes in clumps, within the eosinophilic globules. Twelve and 20 days after injection of tritiated thymidine, some of these ingested spermatozoa are labeled. Therefore, the nutritive phagocytes ingest not only relict spermatozoa, but also some newly-formed spermatozoa, and sometimes spermatids and spermatocytes.

A typical testis from the sample taken September 3, 1963, resembles a typical testis from the previous month, except the basal band of germ cells is thicker. As in the previous month, many primary spermatogonia, maturing spermatogonia, and spermatocytes are labeled by a one-hour exposure to tritiated thymidine. In testes from urchins injected with tritiated thymidine on September 3 and killed three days later, only primary spermatogonia, maturing spermatogonia, and spermatocytes are labeled. Six days after injection, the label has reached the younger, spherical spermatids. In testes from urchins injected on September 3 and kept in the laboratory for 20 days, continuous trails of spermatids and spermatozoa lead, in the spaces between the nutritive phagocytes, from the basal band of germ cells to the lumen, which contains a small mass of spermatozoa (see Fig. 8). Thus, by this time, at least some of the spermatozoa in the lumen are newly-produced and not from a past reproductive cycle. This conclusion, based on anatomical evidence, is borne out by autoradiograms showing labeled spermatozoa in the central sperm mass 20 days after injection. In the same testes, some nutritive phagocytes contain ingested labeled spermatozoa. In testes sampled 40 days after the September 3 injection, the lumen contained labeled spermatozoa, while some ingested clumps of labeled spermatozoa are still seen in the nutritive phagocytes. In testes sampled 100 days after the September 3 injection, labeled spermatozoa are still present in the lumen. After 100 days, no labeled spermatozoa are found in the cytoplasm of the nutritive phagocytes, but labeled amorphous basophilic granules are sometimes present; presumably these labeled granules are the remains of partially digested, labeled spermatozoa.

In typical testes from the samples of October 4 and November 3, 1963, the acinar lumen contains a mass of newly-produced spermatozoa (Figs. 8 and 9). A broad band of primitive spermatogonia, maturing spermatogonia and spermatocytes occupies the basal part of the germinal layer. Streams of spermatids and spermatozoa are proceeding between adjacent nutritive phagocytes toward the lumen. A one-hour exposure to tritiated thymidine labels many primitive spermatogonia, maturing spermatogonia, and spermatocytes. In testes from urchins injected with tritiated thymidine on November 3 and killed six days later, the label has reached the younger, spherical spermatids. Twenty days after the injection of November 3, large numbers of labeled spermatozoa are found in the central sperm mass. Twenty days after injection, no labeled spermatozoa can be detected in the cytoplasm of the nutritive phagocytes. Apparently the phagocytosis of spermatozoa by the nutritive phagocytes of the testes ceases between September and November. The cessation of phagocytosis at approximately the same time that spermatozoa begin to accumulate in the lumen suggests that the nutritive phagocytes may play an important part in regulating the rate of accumulation of spermatozoa in the lumen. Testes from urchins injected on November 3, 1963,

still contain labeled spermatozoa on March 7, 1964 (135 days later). Apparently these urchins, which had an average gonad index of 17.1 at sacrifice, failed to spawn in the laboratory during the first part of January, 1964, when the urchins in the field spawned.

In typical testes from urchins sampled on December 1, 1963, and January 1, 1964, the acinar lumen is distended with a great mass of spermatozoa. The germinal layer is markedly thinner than in earlier months, partly due to shrinkage of the nutritive phagocytes (which have lost most or all of their eosinophilic globules), and partly due to a thinning of the basal band of germ cells. Streams of spermatids and spermatozoa are still proceeding between nutritive phagocytes toward the lumen. In the testes of some individuals (Fig. 10), deglobulated nutritive phagocytes occur among the spermatocytes, maturing spermatogonia, and primitive spermatogonia. Some of these nutritive phagocytes appear to detach parts of the basal band of germ cells from the basement membrane which separates the germinal epithelium from the connective tissue-muscle layer. As in previous months, primitive spermatogonia, maturing spermatogonia, and spermatocytes are labeled by exposure to tritiated thymidine for one hour. In testes from urchins injected with tritiated thymidine on January 1, 1964, and killed six days later, the label has reached the spermatids. Twenty days after injection, there are numerous labeled spermatozoa in the acinar lumen. No testes were available from the urchins injected on January 1, 1964, and sacrificed on March 7, 1964, since all were females.

In a typical testis from urchins sampled on January 30, 1964, several weeks after spawning (A. L. Lawrence, personal communication), the acinar lumen contains only a small number of spermatozoa. The germinal layer still consists of deglobulated nutritive phagocytes and a basal region of germ cells. Many nutritive phagocytes occur among the basal germ cells and divide them into separate clusters of cells, which remain attached by one or two cells to the basement membrane. In many clusters, the most basal cells are primitive spermatogonia (Fig. 11). It is most likely that these cells are the very spermatogonia from which will spring the germ cells of the next annual reproductive cycle. Treatment of such a testis for one hour with tritiated thymidine labels many of the maturing spermatogonia and spermatocytes in the cell clusters (Fig. 12). The primitive spermatogonia at the base of the cell clusters are labeled only rarely.

Typical testes from urchins sampled on February 27, 1964, and on March 22, 1963, have deglobulated nutritive phagocytes and clumps of germ cells at all levels in the germinal layer (Fig. 13). Most of these clumps of maturing spermatogonia and spermatocytes have become detached from the base of the germinal layer and appear to be moving toward the acinar lumen. The lumen contains spermatozoa, often mixed with clumps of spermatids and spermatocytes. A few scattered primitive spermatogonia remain near the base of the germinal layer. Exposure of such a testis to tritiated thymidine for one hour labels many of the maturing spermatogonia and spermatocytes, even as they are progressing toward the lumen in disorganized clumps few of the primitive spermatogonia are labeled. In testes from urchins injected with tritiated thymidine on March 22, 1963, and killed 11 days later, the acinar lumen contains some labeled spermatozoa. Therefore, spermatozoa are produced after spawning has occurred, and the term *relict*

spermatozoa should not necessarily connote spermatozoa produced before spawning. Some labeled spermatozoa were detected in the testes of urchins injected on March 22, 1963, and killed at increasing time intervals (up to 193 days) thereafter.

The ovary. During the 1963–1964 annual reproductive cycle, germ cells were synthesizing DNA in typical ovaries sampled from March through October. These germ cells, which include oogonia and primary oocytes, occur in small groups or nests near the base of the germinal layer. In most ovaries, these cell nests are inconspicuous, and several acinar cross-sections must usually be searched before a nest is located. The oogonia have scanty, clear cytoplasm and a nucleus 5 μ in diameter; the oogonial nucleus contains one or two indistinct nucleoli and little chromatin. The pre-leptotene primary oocytes have a thin shell of clear cytoplasm and a nucleus 4 μ to 5 μ in diameter; a nucleolus is often present and the chromatin is organized into fine threads. The cell nests also contain primary oocytes from the leptotene through the diplotene stages of the first meiotic prophase; the chromatin of these oocytes is organized into a spireme. After the primary oocytes complete the diplotene stage, they enter the interphase-like dictyotene stage. Tennent and Ito (1941) referred to the dictyotene stage as the *diffusion stage* plus the *resting nucleus stage.* The primary oocytes remain in the dictyotene stage until they are fully grown and ready to undergo maturation divisions; only then does the first meiotic prophase terminate. The smallest primary oocytes in the dictyotene stage are scattered at the base of the germinal epithelium.

Exposure to tritiated thymidine for one hour labels some of the oogonia and pre-leptotene primary oocytes in each cell nest [3] (Fig. 14). As early as three days after injection of tritiated thymidine, some of the primary oocytes in the spireme stage have become labeled. In none of the long-term experiments can the label be traced into growing primary oocytes, secondary oocytes and mature ova. Therefore, it may well be that the label remains in the smallest primary oocytes in the dictyotene stage until the following annual reproductive cycle when the labeled primary oocytes grow and undergo maturation divisions. Such an extended dictyotene stage in the human primary oocyte has been reported to last many years (Ohno *et al.,* 1962; Baker, 1963), while a dictyotene stage lasting many months apparently occurs in female mice (Rudkin and Griech, 1962) and in female chicks (Hugres, 1963).

It is convenient to begin a description of the annual cycle of the ovary with specimens of *S. purpuratus* sampled on May 26, 1963. A typical ovary for this stage in the annual reproductive cycle is shown in Figure 15. Most of the acinar lumens contain a few ova from a preceding reproductive cycle (relict ova), some but not all of which are being attacked and digested by deglobulated vacuolated nutritive phagocytes. The germinal layer, in addition to the nutritive phagocytes and germ cell nests already described, has as its base a number of small primary oocytes, apparently produced during the preceding annual reproductive cycle. These elliptical oocytes have a weakly basophilic, fibrous cytoplasm and a

[3] These results are in general agreement with the earlier findings of Nigon and Gillot (1963), who incubated fragments of *Arbacia lixula* ovary in the spring for one hour in sea water containing tritiated thymidine and found a few labeled cells which they interpreted as "jeunes ovocytes" (p. 249). Nigon and Gillot did not attempt to follow the fate of the labeled cells in long-term experiments *in vivo.*

spherical nucleus containing a strongly basophilic, spherical nucleolus. The largest of these primary oocytes is 15 $\mu \times 12$ μ and has a nucleus 9 μ in diameter containing a nucleolus 3 μ in diameter. In typical ovaries (Fig. 16) from urchins collected July 8 and August 7, 1963, the nutritive phagocytes have eosinophilic

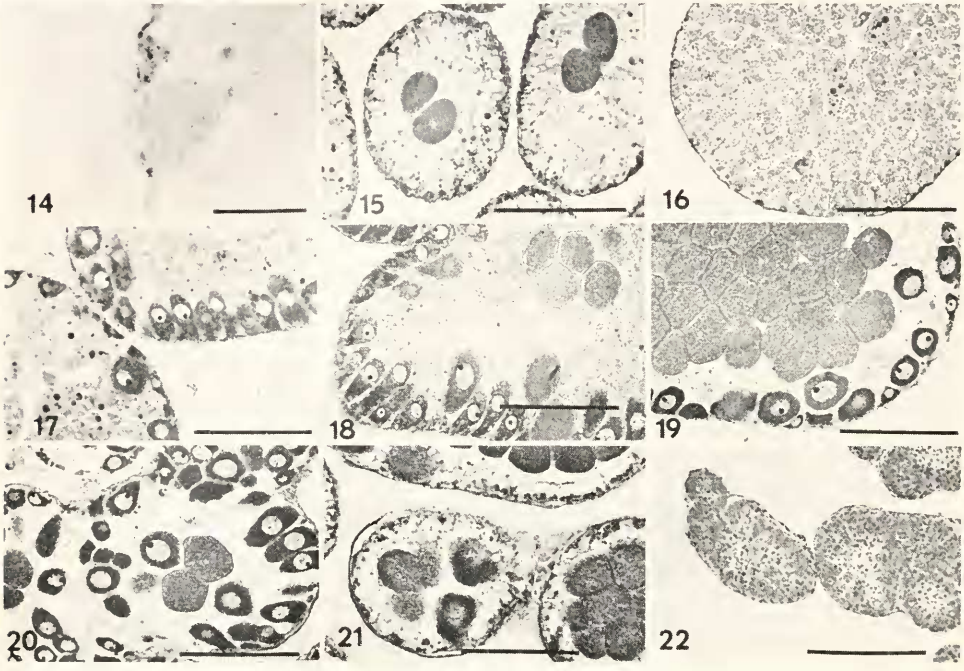

FIGURE 14. An autoradiogram prepared from an ovary of an urchin collected September 3, 1963. Above the large, growing primary oocyte, there is a nest of oogonia and pre-leptotene primary oocytes labeled after exposure to tritiated thymidine for one hour. One of the cells of the visceral peritoneum is also labeled (below the growing primary oocyte). The scale line is 25 μ. Nuclear fast red.

FIGURE 15. Several acini of an ovary from an urchin collected May 26, 1963. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 16. Part of an acinus of an ovary from an urchin collected July 8, 1964. The center of the acinus is at the top. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 17. Parts of two acini of an ovary from an urchin collected October 4, 1963. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 18. Part of an acinus of an ovary from an urchin collected on November 2, 1963. The center of the acinus is at the top right. A primary oocyte of maximum size and an oocyte with a maturation spindle are proceeding, in the spaces between adjacent nutritive phagocytes, toward the acinar lumen. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 19. Part of an acinus of an ovary from an urchin collected on January 1, 1964. The center of the acinus is at the upper right. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 20. An acinus of an ovary from an urchin collected on January 30, 1964. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 21. Several acini of an ovary from an urchin collected on March 22, 1963. Many large oocytes and ova are being digested by deglobulated nutritive phagocytes. The scale line is 150 μ. Hematoxylin and eosin.

FIGURE 22. Several acini of an ovary from a very small (0.3 g.), immature female specimen of *S. purpuratus*. A number of 15 $\mu \times 12$ μ primary oocytes may be seen. The scale line is 150 μ. Hematoxylin and eosin.

globules in their cytoplasm, and relict ova are only rarely seen in the acinar lumens. In all other respects, these ovaries resemble the typical ovary from the sample of May 26, 1963.

In typical ovaries from urchins collected on September 3, 1963, germ cell nests are still present, but many of the primary oocytes at the base of the germinal epithelium are larger and have a more basophilic cytoplasm than in the previous months. The largest of these oocytes is 30 $\mu \times 25$ μ; each has a nucleus 15 μ in diameter containing a nucleolus 5 μ in diameter. In typical ovaries sampled on October 4, 1963, the growing primary oocytes with strongly basophilic cytoplasm continue to lie at the base of the germinal epithelium (Fig. 17). The largest of these oocytes is 45 $\mu \times 30$ μ and has an elliptical nucleus 22 $\mu \times 18$ μ containing a nucleolus 6 μ in diameter. October is the last month in which the germ cell nests occur at the base of the ovarian germinal epithelium.

Typical ovaries sampled November 2, 1963, contain many primary oocytes of maximum size, some at the base of the germinal epithelium and others proceeding between the nutritive phagocytes toward the lumen (Fig. 18). A primary oocyte of maximum size (which many embryologists term an "immature egg" or an "egg in the germinal vesicle stage") is usually elliptical (65 $\mu \times 45$ μ) and has weakly basophilic cytoplasm; the nucleus (or germinal vesicle) is 28 $\mu \times 22$ μ and contains a nucleolus 8 μ in diameter. Some of the oocytes of maximum size (secondary as well as primary) contain maturation spindles (Fig. 18) and are giving off polar bodies. Maturation can occur at any level in the germinal epithelium or in acinar lumen. The acinar lumen contains a few newly-produced ova, which are 60 μ in diameter and each contains a nucleus 9 μ in diameter. Since the diameter of a living ovum of *S. purpuratus* is about 80 μ, it is evident that fixation and embedding causes considerable shrinkage. At the base of the germinal epithelium of ovaries sampled in November, there are growing primary oocytes of assorted sizes and also a number of small cells resembling the oogonia described previously. It is not clear whether these cells are oogonia, very small primary oocytes in the dictyotene stage, or both; they are not labeled by a one-hour exposure to tritiated thymidine. Typical ovaries from the urchins sampled on December 1, 1963, resemble the typical ovaries of the previous month, but have far more ova in the acinar lumens.

The typical ovaries sampled on January 1, 1964, contain more ripe ova than ovaries sampled December 1, 1963, and the nutritive phagocytes have become deglobulated (Fig. 19). In a typical ovary from urchins sampled on January 30, 1964, several weeks after spawning, the acinar lumen contains only a small number of ova, while the germinal layer is much the same as it was on January 1, 1964. Some primary oocytes of maximum size and some oocytes in the process of maturation are still present (Fig. 20). It is, therefore, very likely that large primary oocytes continue to mature into ova after spawning has occurred. The large oocytes and the mature ova show no signs of being digested by the nutritive phagocytes.

The ovaries sampled on February 27, 1964, unlike ovaries of the preceding months, contain no medium-sized primary oocytes. At the base of the germinal epithelium are only small primary oocytes, the largest of which is 20 $\mu \times$ 15 μ, each with a nucleus 12 μ in diameter containing a nucleolus 4 μ in diameter. The acinar lumens contain some ova, a number of primary oocytes of maximum

size, and a few oocytes in the process of maturation. A few of these ova and oocytes are being attacked and digested by the nutritive phagocytes. The disappearance of the medium-sized oocytes may be due to the completion of growth and maturation by all oocytes larger than some critical size (which is apparently 20 $\mu \times 15$ μ), while all oocytes smaller than this remain, without growing, at the base of the germinal epithelium.

Spawning in 1964 occurred as in 1963, during the month of January (A. L. Lawrence, personal communication). Therefore, gonads sampled in late March of 1964 and in late March of 1963 should be at a similar stage of development. In ovaries sampled March 22, 1963, many of the ova and oocytes of maximum size are being attacked and destroyed by the nutritive phagocytes (Fig. 21). Where the attacking nutritive phagocytes come into contact with an ovum or oocyte, the boundary of the attacked cell becomes indistinct and its cytoplasm appears to be eroded away. At the base of the germinal layer, the germ cell nests are again present. These nests are probably reconstituted by mitosis of reserve oogonia carried over from the preceding fall. As in the previous month, the largest primary oocytes are still 20 $\mu \times 15$ μ and each has a nucleus 12 μ in diameter containing a nucleolus 4 μ in diameter.

A typical ovary from urchins sampled on April 21, 1963, has only a few ova remaining from the preceding oogenesis and no primary oocytes of maximum size left in the acinar lumens. The nutritive phagocytes continue to attack some of these ova and also appear to be destroying some of the larger (20 $\mu \times 15$ μ) primary oocytes at the base of the germinal layer. By May 26, 1963, when the sample which began this description of the annual ovarian cycle was taken, the largest primary oocytes remaining at the base of the germinal layer are 15 $\mu \times$ 12 μ; each has a nucleus 9 μ in diameter containing a nucleolus 3 μ in diameter.

Immature gonads. In all specimens of *S. purpuratus* weighing less than 3.0 g. (16 mm. in test diameter), the gonads remain immature throughout the year, even at the height of the reproductive season for the majority of the population. Although Fuji (1960) reported that the gonads of small specimens of *Strongylocentrotus intermedius* are neuter, the sex of small specimens of *S. purpuratus* can be determined by microscopic investigation of their gonads. The germ cells of immature ovaries include oogonia, pre-leptotene primary oocytes, primary oocytes in the spireme stages of the first meiotic prophase and primary oocytes in the dictyotene stage. The largest of these dictyotene primary oocytes is 15 $\mu \times 12$ μ and has a nucleus 10 μ in diameter containing a nucleolus 3 μ in diameter (Fig. 22). Exposure of the immature ovaries to tritiated thymidine for one hour labels many of the oogonia and pre-leptotene primary oocytes. The primary oocytes which were produced on June 15, 1963, were presumably those which would grow and mature during the 1964–1965 reproductive cycle. The only germ cells in the testes of immature urchins are apparently primitive spermatogonia. A one-hour exposure to tritiated thymidine labels some of the primitive spermatogonia in these testes.

Discussion

The present investigation has elucidated the time course of the later events of spermatogenesis in *S. purpuratus*. The interval from primary spermatocyte DNA synthesis to early spermatid is about six days. It is probable that each

primary spermatocyte spends most of this six-day period in an extended first meiotic prophase, and then divides into two secondary spermatocytes, each of which quickly divides into two early spermatids. Since the first labeled spermatozoa appear about 10 days after labeling, approximately four days are required for a spermatid to differentiate into a mature spermatozoon (the process of spermiogenesis). The duration of the later phases of spermatogenesis in the sea urchin is comparable with those in the fruit fly, where the interval from primary spermatocyte DNA synthesis to early spermatid is five days and where spermiogenesis takes three days (Chandley and Bateman, 1962). These intervals in both the sea urchin and the fruit fly are considerably shorter than in the laboratory rat. In the rat, the interval from primary spermatocyte DNA synthesis to early spermatid is 18 days, while the interval from early spermatid to ripe spermatozoan is approximately 17 days (Clermont *et al.*, 1959).

In *S. purpuratus*, the time-course for the later events of spermatogenesis was the same for the production of spermatozoa in August, September, November, January, and March of the 1963–64 annual reproductive cycle. This uniformity in the duration of the cellular transitions between primary spermatocyte DNA synthesis and the appearance of mature spermatozoa means that this part of spermatogenesis can exert no control over the rate of sperm production. Therefore, the events which control the rate of sperm production must occur prior to the termination of DNA synthesis by the primary spermatocytes. The nature of these controlling events was not elucidated in the present investigation. Several possible schemes may be proposed to explain the seasonal fluctuations in the rate of sperm production. In the simplest of these, sperm production could be controlled by seasonal fluctuations in the duration of the growth-duplication cycle of the primitive spermatogonia. For example, in the spring the average duration of the growth-duplication cycle could be several weeks or months, but during the late summer and fall it may shorten to about one day.

In *S. purpuratus*, the rate of sperm production does not necessarily equal the rate of sperm accumulation in the lumen of the testis. Early in the ripe season, newly produced spermatozoa are often ingested by the nutritive phagocytes, and never reach the lumen. Therefore, the rate of sperm accumulation in the lumen is determined both by the rate of sperm production and by the phagocytic activity of the nutritive phagocytes.

In the ovary of *S. purpuratus*, it is probable that the rate of increase in size of the primary oocytes (and not the rate of their production the year before) determines the rate of production of ripe ova. The possibility that the nutritive phagocytes of the ovary exert a partial control over production of ova was neither proved nor disproved in the present investigation. Since, in any given year, there is no significant difference in the gonad index curve of male and female specimens of *S. purpuratus* (Bennett and Giese, 1955), it is possible that the same factor or factors which influence spermatogonial proliferation may influence oocyte growth in the female.

For a number of years, the annual reproductive cycle of *S. purpuratus* has been studied by monthly determinations of the gonad index of a periodically-sampled population of urchins (Giese, 1959). The shape of the gonad index curve and the time of spawning are often strikingly different from one year to the next. Spawning during the 1963–1964 annual reproductive cycle studied was

in January of 1964. Spawning of the Yankee Point population of *S. purpuratus* has been as late as May (Bennett and Giese, 1955). At present, it is not known whether the time-course of germ cell proliferation and growth in years of early spawning and years of late spawning is different.

The nutritive phagocytes, in addition to the phagocytic activity by which they destroy relict gametes and control the accumulation of spermatozoa, also have a nutritive function in *S. purpuratus*. The cytoplasmic globules probably arise in part from ingested gametes and in part from nutrients translocated to the gonads by the coelomic fluid and hemal system. The cytoplasmic globules probably have a complex composition; a conspicuous component is a neutral mucopolysaccharide-protein complex (Holland, 1964). In the gonads of *S. purpuratus*, the globules of reserve abruptly disappear as the gonads approach maturity nearing the height of the reproductive season. Even as the globules disappear, the gonad index continues its steady rise due to the rapid increase in cell number in the testis and in the cell size in the ovary. The direct phagocytosis of globules from nutritive phagocytes by growing oocytes, reported for *Arbacia punctulata* by Liebman (1950), was never observed in the ovaries of *S. purpuratus*. Therefore, the reserves stored in the nutritive phagocytes are presumably translocated in a soluble form to the germ cells (and possibly, to some extent, to other tissues as well). The globules in the nutritive phagocytes apparently were not utilized until most of the reserves stored in the inner epithelium of all major gut regions had been depleted during October and November of 1963 (A. L. Lawrence and J. M. Lawrence, personal communication). It is probable that a large part of these gut reserves was translocated to the gonads to support germ cell growth and proliferation early in the ripe season.

Summary

1. The DNA-synthesizing cells in the gonads of the purple sea urchin were labeled with tritiated thymidine and detected with autoradiography.

2. Some fibroblasts in the connective tissue-muscle layer and some cells of the visceral peritoneum covering the gonads synthesize DNA.

3. Some of the non-germinal cells (the nutritive phagocytes) of the germinal layer proliferate during the spring and early summer, but proliferation ceases in the late summer as the nutritive phagocytes begin to accumulate cytoplasmic globules of reserve. Nutritive phagocytes labeled with tritiated thymidine in their deglobulated phase in the spring are still labeled up to several months later, after they have acquired cytoplasmic globules.

4. In the testes of male urchins at the beginning of the reproductive season, not only relict spermatozoa, but also some newly-formed spermatozoa are ingested by the nutritive phagocytes. The phagocytosis of newly-formed spermatozoa ceases later in the reproductive season, at approximately the same time that spermatozoa begin to accumulate in the lumen.

5. In the germinal epithelium of the testis, the germ cells which synthesize DNA are the spermatogonia and primary spermatocytes. Primary spermatocytes labeled with tritiated thymidine differentiate into early spermatids in about six days, while spermiogenesis takes approximately four days.

6. Throughout the portion of the annual reproductive cycle when spermatozoa are being produced (August through March), the time course for the later events of spermatogenesis remains constant. Therefore, the events which control the rate of sperm production must occur early in spermatogenesis.

7. In the germinal layer of the ovary, the germ cells which synthesize DNA are the oogonia and the pre-leptotene primary oocytes. During the same annual reproductive cycle, long-term experiments failed to demonstrate the differentiation of pre-leptotene primary oocytes into growing primary oocytes, maturing oocytes or ova. Therefore, it is likely that labeled primary oocytes remain small and inconspicuous until the following annual reproductive cycle when they grow and mature into ova.

8. The sex of very small, immature urchins may be determined by histological examination of their gonads. The ovaries of immature female urchins contain small primary oocytes which are presumably the source of ova developing during the first annual reproductive cycle.

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